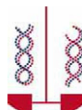
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Nuclear localization of reporter genes activated by curved DNA

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Curved DNA structures with a left-handed superhelical conformation can activate eukaryotic transcription. Mechanistically, these structures favor binding to histone cores and can function as a docking site for sliding nucleosomes. Thus, promoters with this kind of curved DNA can adopt a more open structure, facilitating transcription initiation. However, whether the curved DNA segment can affect localization of a reporter gene is an open question. Localization of a gene in the nucleus often plays an important role in its expression and this phenomenon may also have a curved DNA-dependent mechanism. We examined this issue in transient and stable assay systems using a 180-bp synthetic curved DNA with a left-handed superhelical conformation. The results clearly showed that curved DNA of this kind does not have a property to deliver reporter constructs to nuclear positions that are preferable for transcription. We also identify the spatial location to which electroporation delivers a reporter plasmid in the nucleus.

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[**Key words:** Chromatin engineering; Gene expression; Curved DNA; Gene localization; Cell nucleus; Transgene; Electroporation]

Regions of curved or bent DNA are found in a variety of cellular and viral genomes from bacteria to man. They are frequently found in biologically important regions such as origins of DNA replication, regions that regulate transcription, and recombination hot spots. An understanding of the essential roles of these regions was largely obtained in the late 1980s and 1990s (1–3). In eukaryotic transcription, curved DNAs function in several ways. These include organization of local chromatin structure to increase the accessibility of *cis*-DNA elements; juxtaposition of the basal transcription machinery with effector domains on upstream-bound factors; regulation of transcription in association with transcription-factor-induced bending of DNA; and accepting nucleosomes sliding from the core promoter region (4,5).

Based on this knowledge, synthetic curved DNAs have been prepared to activate transcription artificially. In these DNAs, dT/dA tracts (a run of several dT/dA base pairs) with a periodicity of about 10 bp form a curved DNA structure, and a slight difference in the periodicity is reflected in the three-dimensional (3D) architecture of the structure: i.e., periodicities of 9, 10 and 11 bp form left-handed superhelical, planar, and right-handed superhelical curvatures, respectively. Thus, a three-dimensional curved architecture can be designed by changing the periodicity of the dT/dA tracts. We have found that curved DNA structures with a left-handed superhelical conformation can activate transcription. Thus, the sequences T4, T8, T12, T16, T20, T24, T28, T32, T36 and T40, in which T indicates a $(dT/dA)_5$ tract and the numeral indicates the number of tracts, all activate transcription in a transient transcription assay system in COS-7 cells (6,7).

The activation potentials of T4, T12, T20, T24, T28, T32, T36 and T40 were further studied in various systems (5,7–10). T12, T20, and T28 were used in a yeast minichromosome system and all activated transcription by 50- to 60-fold (5). The potential transcriptional activation of T20 was also investigated in a genome chromatin context in HeLa cells, mouse embryonic stem (ES) cells and hepatocytes differentiated from the ES cells (7,9). In HeLa cells, T20 activated transcription of a reporter gene positioned adjacent and downstream of the curved DNA, but in the genomes of ES cells and hepatocytes the activation was dependent on the locus of integration. These results suggest that in cells that have a strict gene regulation system, transcriptional activation by T20 occurs only in a transcriptionally active locus in the genome. T4-, T20-, T24-, T28-, T32-, T36- and T40-linked reporter gene constructs were introduced into mouse liver from the tail vein using a rapid, high-volume injection method (hydrodynamics-based administration), which enables delivery of the construct DNA into nuclei of liver cells. Transcriptional activation by the T segments was roughly 2- to 8-fold *in vivo* (8,10).

The spatial position of a given gene in the nucleus is an important parameter in its expression (11–16). The synthetic left-handed curved DNA segments (referred to as Tn segments below) may influence the localization of reporter constructs and this effect, if present, may also be involved in transcriptional activation by these segments. A clearer understanding of this issue may make it possible to develop a more efficient transgene expression system. Therefore, the current study

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was performed to investigate whether a spatial location-dependent effect is involved in transcriptional activation by Tn segments.

MATERIALS AND METHODS

Reporter constructs, cells and culture conditions The structures of the reporter constructs, pST0/TLN-7 and pLHC20/TLN-6, have been reported previously (6,7). These constructs were introduced into COS-7 or HeLa cells by electroporation (6,7), after which the cells were cultured on 60-mm dishes for 12 h. The cells were then washed 3 times with phosphate-buffered saline (PBS) (−), trypsinized, and suspended in a small volume of fresh medium. They were then transferred onto glass slides, cultured for 12 h, and subjected to fluorescence *in situ* hybridization (FISH) analyses.

COS-7, HeLa and their derivative cell lines were grown in Eagle's minimum essential medium containing 5% fetal bovine serum at 37°C in 5% CO_2. Mouse ES cell line E14TG2a (17) and its derivative cell lines were grown in Glasgow minimum essential medium supplemented with 10% fetal bovine serum, 0.1 mM 2-mercaptoethanol, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 1000 units/ml of leukemia inhibitory factor (Chemicon, Temecula, CA, USA) on gelatin-coated dishes without feeder cells at 37°C in 7.5% CO_2. Then, 6×10^4 cells were transferred onto glass slides, cultured for 12 h, and subjected to FISH analyses.

DNA probe DNA probes were prepared by nick translation of pST0/TLN-7 (6), pLHC20/TLN-6, pLHC20/*loxP*/TLN-6 (7) and pLHC20/*loxP*/*neo*/SL (9) and labeled with biotin-16-dUTP or digoxigenin-11-dUTP (Roche Diagnostics GmbH, Mannheim, Germany). The conditions for these reactions were those recommended by the manufacturer. The probe solutions at a final concentration of 30 ng/μl contained 50% formamide, 10% dextran sulfate and 2× SSC.

FISH Cell fixation, permeabilization, freeze/thaw treatment and HCl/pepsin treatment were performed as described previously (18–21). After HCl/pepsin treatment, cells were fixed again in 1% paraformaldehyde/PBS (−) for 10 min, washed with PBS (−) and 2× SSC for 5 min, and stored in 50% formamide/2× SSC at 4°C overnight. Cells were then immersed sequentially in 70%, 90% and 100% ethanol at room temperature for 2 min. Denaturation was performed in a solution containing 70% formamide and 2× SSC at 73°C for 5 min, after which the cells were incubated in 70% ethanol at −30°C for 5 min. Subsequently, the cells were immersed again sequentially in 70%, 90% and 100% ethanol at room temperature for 2 min. Hybridization was performed with the probes described above in a humid chamber at 37°C for 3 days. The cells were then washed three times with 0.1× SSC at 60°C for 5 min and blocked for 30 min in 4× SSC solution containing 5% BSA and 0.05% Tween-20. Biotin-labeled probes were detected using FITC-avidin (Invitrogen, Carlsbad, CA) and the signals were amplified with biotinylated anti-avidin antibody (Vector Laboratories, Inc., Burlingame, CA) and FITC-avidin. Digoxigenin-labeled probes were detected with rhodamine-conjugated anti-digoxigenin Fab fragments (Roche). Samples were mounted in VECTASHIELD Mounting Medium with DAPI (Vector Laboratories).

Cell differentiation Mouse ES integrants were differentiated into hepatocytes using the procedure described by Tanase et al. (9), with a slight modification in step 3 of the reported procedure, in which we cultured the cells on collagen-coated coverslips (thickness 0.17±0.02 mm). Differentiation into hepatocytes was confirmed by immunostaining for cytokeratin-18 (CK18), a hepatocyte-specific gene product. FISH and immunostaining were performed simultaneously. The primary and secondary antibodies used to detect CK18 were goat anti-mouse CK18 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and Alexa Fluor 647 donkey anti-goat IgG (Invitrogen), respectively. Only CK18-positive cells were used for data acquisition for hepatocytes.

Image acquisition, processing and analysis Images were collected using an Olympus FluoView FV1000-D confocal microscope equipped with a UPlanSApo 60× O (NA=1.35) or a PlanApoN 60× OSC (NA=1.4) objective lens. 3D stacks were typically obtained with 70 steps of 0.2 μm. For chromatic aberration, an *xyz* correction was performed based on bead measurements. The evaluation of relative distances between the nuclear center and FISH signals was performed as follows. Images were processed using MetaMorph software (Molecular Devices, Inc.) with a 4D viewer plug-in. First, Gaussian filtering (3×3) and median filtering (5×5) were applied to remove noise from the images. Dark spots that were not stained with DAPI were subjected to "smoothing" using a function (Morphology Filters) in the software. Reasonable thresholds were then chosen to define the border of the nucleus, and the nuclear center and the centers of FISH signals were determined. Relative distances between these centers were measured using a custom-written R script.

RESULTS

We first investigated the distribution of each reporter construct in the nucleus of COS-7 cells and HeLa cells, using the pLHC20/TLN-6 and pST0/TLN-7 luciferase gene-based reporter constructs (6,7). pLHC20/TLN-6 harbors the T20 segment (Fig. 1) just upstream of the luciferase gene and activates expression of this gene in COS-7 cells by roughly 70-fold (7). pST0/TLN-7 has a straight DNA segment instead of T20 and was used as a control. In the current study, these constructs were introduced into COS-7 and HeLa cells and cultured using a previously described procedure with a slight modification (6). Localization of the constructs was determined by FISH analysis using a biotin- or digoxigenin-labeled probe that hybridizes to the plasmids. As expected, the number of molecules introduced into a nucleus was variable, but the profiles were similar for pLHC20/TLN-6 and pST0/TLN-7 in COS-7 and HeLa cells respectively (Fig. 2).

Localization of each construct in the nucleus was analyzed by measuring the distance between the FISH signal and the nuclear center. Using three-dimensional stacks with 20 to 40 steps of 0.2 μm, we searched for the locations of signals, determined the distances from the nuclear center, and then sorted these distances into 10 fractions (Fig. 3). The population of each construct in a "shell" gradually increased from the central shell to the outermost shell (Figs. 3A–D). This profile corresponds to the pattern generated by random distribution. Thus, the constructs introduced into a nucleus seemed spread uniformly in a nucleus. Obviously, the presence of T20 did not cause any substantial difference in the plasmid distribution in COS-7 and HeLa cells (Figs. 3A–D).

The spatial location of the reporter in the nucleus was subsequently studied using three sets of HeLa cell integrants: HLB8 and HLB8/T20, HLB10 and HLB10/T20, and HLB13n5 and HLB13n5/T20, where each set is a pair of a T20-less control cell line and a T20-containing cell line. The locus of the reporter integration is the same within a set (7). Fig. 4A shows the effect of T20 on transcription, which was reported by Sumida et al. (7) and shown here again. We also used FISH for analysis of localization. As shown in Figs. 4B and C, the profiles of the reporter distribution in the nucleus did not differ significantly between HLB8 and HLB8/T20, between HLB10 and HLB10/T20, or among these four cell lines, as judged by the Kolmogorov–Smirnov test (22). On the other hand, regarding the set, HLB13n5/T20 and HLB13n5, the reporter gene localized closer to the nuclear periphery in the former than in the latter (Fig. 4C; $p=0.004$).

The same analysis was performed using four sets of mouse ES integrants: MES32 and MES32/T20, MES25 and MES25/T20, MESA2 and MESA2/T20, and MES7 and MES7/T20. Each set comprises a T20-less control cell line and a T20-containing cell line and the locus of reporter integration is the same within a set (9). In these sets, transcriptional activation by T20 is weak (9), which is shown in Fig. 5A for reference. Except for MES32 and MES32/T20, localization of the reporter was not influenced by the presence of T20 (Figs. 5B and C). In MES32/T20, the reporter location was closer to the nuclear periphery compared with MES32 (Fig. 5C).

The effect of differentiation on the spatial location of the reporter was also investigated. ES cells were differentiated into hepatocytes using a method described previously (9) (Fig. 5B). The cytokeratin-18 (CK18), which is a hepatocyte-specific gene product, is stained in green in the hepatocyte images, and thus confirming the completion of the differentiation. As shown in Fig. 5C, during the differentiation, spatial location of the reporter gene slightly shifted inward toward the center of the nucleus in each cell line. However, the relative radial positions of the reporter gene in the nucleus were almost the same between the test and control cell lines (Fig. 5C, the right panel).

DISCUSSION

In the current study, we examined whether T20 influences the localization of reporter constructs in the nucleus to determine whether a spatial location-dependent effect is involved in transcriptional activation by T20-containing segments.

The T20 did not influence efficiency of the plasmid introduction into a cell nucleus (Fig. 2) and it did not cause any difference in the plasmid distribution in a nucleus (Fig. 3). Thus, these results clearly show that T20 did not exert any effect on reporter localization in the transient assay systems. In other words, the T20-dependent

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